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Natural phytosanitary products effects on *Bacillus thuringiensis* subsp. *kurstaki* (Berliner)

Efeito de produtos fitossanitários naturais sobre *Bacillus thuringiensis* subesp. *kurstaki* (Berliner)

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Abstract

This work aimed to evaluate the effect of natural phytossanitary products (NPP) on spores and crystal toxicity of *Bacillus thuringiensis* subsp. *kurstaki* – HD1 (Btk). For this commercial products (Agromos, Biogermex, Bovemax, Bordeaux mixture, Ecolife®, Dalneen, Matan Plus, Pyronin and Stüble-Aid®) were used at three different concentrations. The effect of NPP on spores was assessed by comparing a suspension of Btk + NPP with sterile distilled water (SDW) and another suspension with nutrient broth (NB), inoculated on nutrient agar (NA) in Petri dishes to quantify the number of CFU/mL, 18 h after inoculation and incubation. The effect of NPP on crystals was evaluated with a suspension of Btk+SDW+NPP added to the artificial diet supplied for *Anticarsia gemmatalis* Hub. (Lepidoptera: Noctuidae) quantifying the number of dead larvae at 12, 24, 48 and 72 h. Matan Plus was the only natural product that did not present effect on spores. All other products, regardless of concentration, decreased significantly CFU/mL. Regarding crystals, Bordeaux mixture was the only one that reduced significantly Btk insecticidal activity at three concentrations.
Key words: Alternative control, entomopathogenic bacteria, integrated pest management

Resumo

Este trabalho objetivou avaliar o efeito dos produtos fitossanitários naturais (PFN) sobre esporos e sobre a toxicidade dos cristais de *Bacillus thuringiensis* subesp. *kurstaki* – HD1 (Btk). Para tal foram usados os produtos comerciais (Agromos, Biogermex, Bovemax, Calda Bordalesa, Ecolife®, Dalneen, Matan Plus, Pironin e Stuble – Aid®) em três diferentes concentrações. O efeito dos PFN sobre esporos foi avaliado comparando-se suspensões de Btk + PFN com água destilada esterelizada (ADE) e suspensões com caldo nutriente (CN), inoculadas em ágar nutriente (AN) em placas de Petri, quantificando-se o número de unidades formadoras de colônias (UFC / mL), 18 h após a inoculação e incubação. O efeito dos PFN sobre cristais foi avaliado com suspensões de Btk + ADE + PFN adicionados à dieta artificial fornecida para *Anticarsia gemmatalis* Hub. (Lepidoptera: Noctuidae), quantificando-se o número de lagartas mortas nos tempos 24, 48 e 72 h. Matan Plus foi o único produto natural que não apresentou

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efeito sobre esporos. Todos os outros produtos, independentemente da concentração, diminuíram significativamente as UFC/mL. Com relação aos cristais, a Calda Bordalesa foi o único produto que reduziu significativamente a atividade inseticida de Btk nas três concentrações.

Palavras-chave: Controle alternativo, bactérias entomopatogênicas, manejo integrado de pragas

Introduction

Phytossanitary products are often applied in conventional or alternative agricultural systems with the objective to enhance crop yield, however, the excessive and inappropriate use of those products is dangerous to health. Consumers aware of this danger search for "cleaner" food nowadays. Therefore, NPP – natural phytossanitary products – have been the scope of researchers to find alternatives that can ensure agriculture sustainability and consumers demands.

Entomopathogens are important natural factors of the environment that regulate insect-pests populations (LACEY et al., 2001), such as *Bacillus thuringiensis* (Bt) that due to biological characteristics, stands out as the main bacteria used in biocontrol (ANGELO; VILAS-BÔAS; CASTRO-GÓMEZ, 2010) and can be applied alone or in association to phytossanitari products and NPP as formulated biopesticide in traditional and alternative crops systems.

However, the action of microbial control agents can be stimulated, supressed or unchanged after association with phytossanitary products. These products may inhibit growth, reproduction, cause mutations, and even inactivate microorganisms, decreasing virulence to a particular pest (ALVES; MOINHO JUNIOR; ALMEIDA, 1998).

Several studies have been conducted to verify the interaction between chemical products and entomopathogenic bacteria. Different techniques can be used to evaluate the effects of chemical products on spores of *B. thutingiensis* subsp. *thuringiensis* (Btk) such as paper disks impregnation technique with broth products (DOUGHERTY; REICHELDERFER; FAUST, 1971; JIMENEZ; ACOSTA; FERNANDEZ, 1989) and with liquid culture medium (SUTTER et al., 1971, CHEN et al., 1974), and mixing products to the molten culture medium technique from the works of Salerno, Dias and Sagardoy (1999), Batista Filho; Almeida and Lamas (2001), Almeida et al. (2003) and Gassen, Batista Filho and Zappelini (2006). The effects of chemical products on Btk using different techniques was studied by Silva et al. (2006, 2008) and the results varied according to chemical group, technique used, concentration, contact time, and development stage of the pathogen.

Studying natural phytossanitary products, Krischik, Barbosa and Reichelderfer (1988) evaluated the effect of nicotine and rutin on Bt and observed that both reduced growth and germination and only nicotine affected *Manduca sexta* toxicity. When non-sporulating bacteria and plant extracts were studied, rosemary (*Rosmarinus officinalis*) presented negative effect on *Staphylococcus aureus* and *Bacillus cereus* when evaluated at different times and concentrations (GENENA et al., 2008), whereas rue (*Ruta graveolens*) inhibited growth of several bacteria species, including the genus *Bacillus* (PEREIRA et al., 2006; MENDES et al., 2008).

Given the above, further studies are needed concerning phytossanitary products effects on Bt since there are few information about this interactions, especially with NPP considered safer than conventional ones. This study was performed to evaluated the effects of natural phytossanitary products on spores and on toxicity of *B. thuringiensis* subsp. *kurstaki* against *Anticarsia gemmatalis* under laboratory conditions.

Material and Methods

Bacillus thuringiensis subsp. *kurstaki* HD-1 (Btk) obtained from the commercial product Dipel

PM® was used. NPP samples were obtained from commercial products (Table 1) and the evaluations were made with the following concentrations: recommended concentration of the product (RC), half that concentration (½ RC) and twice that concentration (2RC).

Larvae of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) were obtained from the laboratory culture, in containers with artificial diet, according to Hoffmann-Campo, Oliveira and Moscardi (1985), maintained at 26 ± 2 °C, RH: 70% ± 10% and 14 h photoperiod until the larvae reach the second instar.

Table 1. Commercial name, composition, use and recommended concentration of natural products used in the experiments.

Product	Composition	Use	Recommended concentration/ha[1]
Agro-Mos®	Cell wall of *Saccharomyces cerevisiae*/ oligosaccharides	Resistance inducer	1.5L
Biogermex	Citrus bioflavonoids (vitamin P), phytoalexins citric, ascorbic acid (vitamin C), citric acid, polypeptides citric, palmitic acid, various fatty acid, sugars, glicerydes and tocopherols	Bactericide / Fungicide	0.2L
Bovemax	*Beauveria bassiana* conidia Emulsionnable Oil formulation	Insecticide	1L
Bordeaux Mixture	Lime and copper sulfate 1Kg CuSO4 + 1Kg Cal	Fungicide	
Ecolife®	Bioflavonoids, fitoalexins, ascorbic, lactic and citric acids	Resistance inducer	0.75L
Dalneem	*Azadirachta indica* oil seed	Insecticide	1L
Matan Plus	Sulfur and nitrogen	Insecticide	
Pironim	*Azadirachta indica* oil seed, rotenone, pyrethrum, allamanda, Black pepper and pyroligneous extract	Insecticide	0.6L
Stubble-Aid®	Copper sulfate (28.0g/L); Ferrous sulfate (51.6g/L); Manganese sulfate(12.0g/L); Zinc sulfate (42.0g/L)	Biofertilizer	1L

[1] Concentration recommended by the manufacturer, standardized at 100L/hectare.
Source: Elaboration of the authors.

NPP Effect on Btk spores

NPP mixed with sterile distilled water. A bacterial suspension (3.75 × 10^4 spores/mL) in 20 mL of sterile distilled water (SDW) was prepared. This suspension was diluted and aliquots of 300 µL (5.6 × 10^3 spores/mL) were added to Erlenmeyer flasks containing 50 mL SDW mixed with the NPP at the different concentrations.

NPP mixed with nutrient broth. Bacterial suspension was prepared with the same procedure described in the previous section but instead of SDW, nutrient broth (NB) was used.

Five Erlenmeyer flasks (replicates) were used for both experiments and for each NPP tested concentration (treatment). Water was used for the control treatment. Flasks were incubated in horizontal shaker (30 ± 2 ºC, 150 rpm for 2 h) and pH was measured prior and subsequent to incubation. Five points (5µL/point) from each flask were inoculated on the surface of nutrient agar (NA) in three Petri dishes (ALVES; MORAES, 1998). Inoculated Petri dishes were kept open in laminar flow chamber for five minutes to evaporate the excess of water and, after that, dishes were closed and incubated in acclimatized chamber (30 ± 2 ºC) during 18 h. The number of colony-forming units (CFU) per point was counted and the final result was expressed in CFU/mL.

NPP effect on crystal toxicity

Bacterial suspension was prepared with 31.3 × 10^9 spores/mL in SDW. Samples of 10 mL (1.5 × 10^9 spores/mL) from this suspension were transferred to Erlenmeyer flasks containing 40 mL of SDW and NPP at the different concentrations, then these flasks were incubated as described above. The pH was measured prior to incubation and at the end of incubation.

Subsequently, 150 µL aliquots of the suspension (4.5 × 10^7 spores/mL) were added in about 1.5 cm on the side surface of artificial diet cubes for *A. gemmatalis* in Petri dishes that were kept open in laminar flow chamber for about 15 minutes to evaporate the excess of water and, after that, each Petri dish received 25 second-instar larvae of *A. gemmatalis*. All treatments consisted of five replicates, with a Petri dish each one. Identical batches were prepared for control, with larvae receiving NPP at the three tested concentrations and Btk suspension separately in diet. Also, an absolute control group was assembled with larvae supplied with the artificial diet treated only with sterile water. The dishes were placed in acclimatized chamber (26 ± 2 ºC, RH: 70% ± 10% and 14 h photophase) and the evaluations were recorded at 12, 24, 48 and 72 h, quantifying the number of dead caterpillars. Death caused by Btk was confirmed with the observation of larvae symptoms and pathogen signs as described by Habib and Andrade (1998).

Data analysis

Data was submitted to analysis of variance (F test) and means were compared among themselves by Tukey test, both at 5% significance, using the Sisvar software program (FERREIRA, 2007). When needed, data was previously transformed into to be analyzed.

Results and Discussion

NPP Effect on the Btk spores

NPP mixed with sterile distilled water. Stubble-Aid® decreased CFU/mL as concentration increased, whereas Matan Plus did not decrease CFU/mL in any concentration. All other products, regardless of concentration, decreased significantly CFU/mL presenting variation between 12.8% and 100% when compared to control treatment (Table 2).

NPP mixed with nutrient broth. Agro-Mos® decreased CFU/mL as concentration increased, whereas other products presented a significant negative effect ranging from 29.6% to 100% (Table 3).

Table 2. *B. thuringiensis* subsp. *kurstaki* HD-1 CFU/mL means (± SE) from spores obtained from commercial product after incubation (30 ± 2 ºC, 150 rpm, 2 h) with sterile distilled water and alternative products at different concentrations and initial and final pH.

Treatment	Conc.[1]	Average CFU/mL (x10^5)	CFU Rel. Test (%)[2]	pH	
				0h	2h
Control	-	265.1±17.41a	-	6.46	6.61
Agro-Mos®	½RC	168.0±7.88b	-40.8	3.72	3.72
Agro-Mos®	RC	142.4±17.58b	-49.8	3.61	3.55
Agro-Mos®	2RC	47.2±10.84c	-83.4	3.48	3.40
CV (%) =		20.21			
Control	-	279.1±17.12a	-	6.46	6.61
Biogermex	½RC	13.5±3.34b	-95.2	4.08	4.02
Biogermex	RC	0.0±0.00b	-100.0	3.76	3.67
Biogermex	2RC	0.0±0.00b	-100.0	3.48	3.54
CV (%) =		26.7			
Control	-	338.7±12.23a	-	6.46	6.61
Bovemax	½RC	275.5±9.42b	-18.7	5.99	6.79
Bovemax	RC	295.4±11.88b	-12.8	5.97	6.72
Bovemax	2RC	282.7±6.5b	-16.5	5.99	6.55
CV (%) =		7.7			
Control	-	241.2±18.55a	-	6.46	6.61
Bordeaux misture	½RC	31.7±6.75b	-86.8	12.5	12.09
Bordeaux misture	RC	0.0±0.00b	-100.0	12.6	12.28
Bordeaux misture	2RC	0.0±0.00b	-100.0	12.6	12.35
CV (%) =		32.34			
Testemunha	-	333.6±21.6a	-	6.46	6.61
Ecolife®	½ RC	0.0±0.00b	-100.0	3.83	3.80
Ecolife®	RC	0.0±0.00b	-100.0	3.73	3.78
Ecolife®	2RC	0.0±0.00b	-100.0	3.42	3.36
CV (%) =		28.96			
Control	-	305.33±12.89a	-	6.46	6.61
Matan Plus	½ RC	309.6±17.24a	1.4	4.78	5.68
Matan Plus	RC	313.6±9.00a	2.7	5.02	5.53
Matan Plus	2RC	308.0±20.57a	0.9	5.18	5.41
CV (%) =		10.55			
Control	-	233.0±12.76a	-	6.46	6.61
Dalneem	½ RC	2.3±1.29b	-98.7	4.94	4.89
Dalneem	RC	0.0±0.00b	-100.0	4.77	4.84
Dalneem	2RC	0.0±0.00b	-100.0	4.66	4.78
CV (%) =		23.1			
Control	-	262.1±21.55a	-	6.46	6.61
Pironim	½RC	185.1±7.56b	-29.4	3.66	3.58
Pironim	RC	199.2±13.69b	-24.0	3.49	3.46
Pironim	2RC	185.1±7.56b	-29.4	3.31	3.30
CV (%) =		14.89			
Control		282.4±7.49a	-	6.46	6.61
Stubble Aid®	½ RC	223.9±8.95b	-20.7	3.56	3.61
Stubble Aid®	RC	151.2±14.29c	-46.5	3.47	3.40
Stubble Aid®	2RC	49.7±10.53d	-82.4	3.30	3.21
CV (%) =		15.1			

Means (± SE) followed by the same letter in column do not differ significantly by the Tukey test (P <0.05).
[1]Product concentration: ½ RC = Half the recommended concentration, RC = Recommended concentration; 2RC = Twice the recommended concentration. [2]Formula: [(Treatment Average CFU/ml / Control Average CFU/mL × 100) – 100], with positive values to CFU/mL increase and negative values for reduction when compared to the control.
Source: Elaboration of the authors.

Table 3. *B. thuringiensis* subsp. *kurstaki* HD-1 CFU/mL means (± ASE) from spores obtained from commercial product after incubation (30 ± 2 ºC, 150 rpm, 2 h) with nutrient broth and alternative products at different concentrations and initial and final pH.

Treatment	Conc.[1]	Average CFU/mL (x10^5)	CFU Rel. Test (%)[2]	pH	
				0h	2h
Control	-	526.1±26.38a	0.0	7.54	7.47
Agro-Mos®	½RC	212.1±9.99b	-59.7	4.29	4.31
Agro-Mos®	RC	115.5±13.92c	-78.1	4.13	4.07
Agro-Mos®	2RC	18.7±9.13d	-96.5	3.97	3.72
CV (%) =		19.1			
Control	-	565.6±23.31a	0.0	7.54	7.47
Biogermex	½RC	0.0±0.00b	-100.0	7.19	7.18
Biogermex	RC	0.0±0.00b	-100.0	7.16	7.16
Biogermex	2RC	0.0±0.00b	-100.0	7.02	7.20
CV (%) =		18.4			
Control	-	232.5±22.62a	0.0	7.54	7.47
Bovemax	½RC	152.1±9.99b	-34.6	7.30	7.27
Bovemax	RC	157.9±8.62b	-32.1	7.34	7.32
Bovemax	2RC	163.7±9.86b	-29.6	7.34	7.43
CV (%) =		18.83			
Control	-	446.1±21.29a	0.0	7.54	7.47
Bordeaux mixture	½RC	39.5±11.67b	-91.2	11.87	11.66
Bordeaux mixture	RC	0.3±0.27b	-99.9	12.26	11.81
Bordeaux mixture	2RC	0.0±0.00b	-100.0	12.34	12.09
CV (%) =		22.4			
Control	-	562.9±18.13a	0.0	7.54	7.47
Ecolife®	½RC	0.0±0.00b	-100.0	7.24	7.06
Ecolife®	RC	0.0±0.00b	-100.0	7.11	6.94
Ecolife®	2RC	0.0±0.00b	-100.0	6.77	6.70
CV (%) =		14.1			
Control	-	312.5±26.38a	0.0	7.54	7.47
Matan Plus	½RC	288.4±11.6a	-7.7	7.30	7.50
Matan Plus	RC	340.9±11.7a	9.1	7.29	7.22
Matan Plus	2RC	281.8±16.47a	-9.8	7.24	7.14
CV (%) =		28.07			
Control	-	432.7±33.83a	0.0	7.54	7.47
Dalneem	½RC	0.4±0.40b	-99.9	4.94	4.89
Dalneem	RC	0.0±0.00b	-100.0	4.77	4.84
Dalneem	2RC	0.0±0.00b	-100.0	4.66	4.78
CV (%) =		34.9			
Control	-	339.2±15.93a	0.0	7.54	7.47
Pironim	½RC	176.5±14.26b	-48.0	6.23	6.55
Pironim	RC	171.7±16.2b	-49.4	5.34	5.35
Pironim	2RC	147.5±6.95b	-56.5	4.83	4.76
CV (%) =		24.2			
Control		471.3±8.67a	0.0	7.54	7.47
Stubble-Aid®	½RC	72.8±7.60b	-84.6	4.77	4.68
Stubble-Aid®	RC	43.8±2.94c	-90.7	4.22	4.34
Stubble-Aid®	2RC	20.3±4.69c	-95.7	3.98	4.06
CV (%) =		9.4			

Means (± SE) followed by the same letter in column do not differ significantly by the Tukey test (P <0.05).
[1]Product concentration: ½ RC = Half the recommended concentration, RC = Recommended concentration; 2RC = Twice the recommended concentration. [2]Formula: [(Treatment Average CFU/ml / Control Average CFU/mL × 100) – 100], with positive values to CFU/mL increase and negative values for reduction compared to the control.
Source: Elaboration of the authors.

Biogermex and Ecolife® decreased CFU/mL at 100% whereas Dalneem and Bordeaux mixture decreased over 90% regardless of tested concentration, when compared to control treatment. Stubble-Aid® decreased 84.6% at ½ RC, 90.7% at RC and 95.7% at 2RC. Likewise, Pironim presented similar CFU/mL decrease at all concentrations: 48.0%, 49.4% and 56.5%. Still, Bovemax reduced CFU/mL at all tested concentrations ranging from 29.6% to 34.6% (Table 3).

Regardless of incubation time, pH of treatments ranged from 3.21 (Stublle-Aid – 2RC) to 12.35 (Bordeaux mixture – 2RC), observing reduction of CFU/mL both to treatments with acidic pH as well as for to treatments with neutral and basic pH (Tables 2 and 3).

Biogermex and Ecolife® must be emphasized because when mixed with water presented acidic pH and when mixed with NB presented neutral pH similar to the control treatment, and in both cases CFU/mL decreased, indicating that the observed result was caused by some other factor than pH (Tables 2 and 3). According to instructions of manufacturer, Ecolife® consists of bioflavonoids, citric phytoalexins and ascorbic acid, similar composition of Biogermex. Flavonoids and terpenoids are molecules derived from the secondary metabolism of plants, which act as a defense mechanism against insects and pathogens (DIXON; DEY; LAMB, 1983; COWAN, 1999). Moreover, according to Tsuchiya et al. (1996), flavonoids have the ability of binding to cell wall, inactivating them and disrupting membranes.

The decrease of CFU/mL observed from Biogermex and Ecolife® are very noteworthy and may be related to the impediment of germination process or the destructive action on bacterial membrane after germination. Such negative effect on cells can be assured with the study with Ecolife® and cells of *Ralstonia solanacearum* and *Xanthomonas axonopodis* pv. *manihotis* (Gram-negative pathogenic bacteria), the results showed that the inhibition halo was proportional to the increase of product concentration (MOTOYAMA et al., 2003). Similarly, the negative effect caused by Pironim seems not to be related to pH, because pH was close to the control treatment when mixed with NB at ½ RC and CFU/mL also decreased. Thus, this observation may be related to product composition, in which neem (*Azadirachta indica*), rotenone, pyrethrum, allamanda, black pepper and pyroligneous extract are included. According to Venkat et al. (2004) the antimicrobial activity exerted by neem is added by the alkyl piperidine present in black pepper that also has bactericidal property.

Agro-Mos® also caused significant CFU/mL decrease at acidic pH, both when mixed with SDW as with NB (Tables 2 and 3). This product is a resistance inducer, and acts directly on the plant that becomes more tolerant to pests. Due to this mechanism of action, no work has been reported concerning the application of this product directly on any pathogen. The decrease of CFU/mL due to Dalneem may have occurred because this product is composed by neem (*Azadirachta indica*), a plant composed by several constituents such as flavonoids that has antimicrobial action (MOSSINI; KEMMELMEIER, 2005). These results corroborate Alzoreky and Nakahara (2003) who observed *Bacillus cereus* inhibition of growth when exposed to methanol and acetic extracts of neem flowers.

Unlike other products, Bordeaux mixture, presented pH values near 12 when mixed with water or NB. But, in this case, Bordeaux mixture components, such as lime and copper sulfate, may also have contributed to the inhibition of CFU/mL. Hassen et al. (1998) studied the Bt tolerance to copper at six concentrations, and observed that the highest concentration was able to inhibit bacteria growth almost at full. In addition, Gassen, Batista Filho and Zappelini (2006) studied fungicides based on another copper form (copper oxychloride) and also observed Btk incompatibility with them all.

Stubble-Aid®, as well as other liquid bio-fertilizers, is rich in metabolites such as enzymes, antibiotics, vitamins, toxins, phenols, esters and acids that act as nutrient for plants, and still present acaricidal, fungistatic and bacteriostatic activity (GALLO et al., 2002), which may explain the observed negative effect proportional to concentration.

Bovemax, regardless of concentration, reduced the CFU/mL both when mixed with SDW and NB. This product is formulated with conidia of the entomopathogenic fungus *Beauveria bassiana*, vegetable oil and emulsifier. Morris (1975) observed that Bt spores germination decrease when exposed to some emulsifiers. Considering that this fungus does not affect Bt, the emulsifier must have been responsible for the observed negative effects on spores.

There are not specific studies about the effects of natural products on spores of *Bacillus thuringiensis*. The limited information available in literature refers to the effect of natural products on several bacteria regarding vegetative growth , therefore further specific studies are needed to understand the action of these products on Btk spores germination and CFU.

NPP effect on crystal toxicity

Only Bordeaux mixture inhibited crystal toxicity at all concentrations, differing significantly from the control treatment with Btk only. The other products did not affect crystal toxicity related to the total percentage of dead larvae (Table 4). Bovemax, at the three concentrations, Biogermex in the RC + Btk and 2RC + Btk and Agro-Mos® in the CR + Btk caused partial negative effect on the crystal toxicity, with mortality decreased at 48 h, differing only from the control treatment with Btk.

Stubble-Aid® presented positive effect on 2RC + Btk at 48 h, with higher mean of larval mortality percentage, differing significantly from other treatments with Btk (Table 4). Ecolife®, Matan Plus, Dalneem and Pironim caused no adverse effects on crystal toxicity.

Biogermex and Dalneem presented no significant differences in mortality between 48 and 72 h in treatment 2RC + Btk, differently from the absolute control group with Btk and other treatments with Btk, which presented more dead larvae at 48 h (Table 4).

Bovemax at the three concentrations + Btk presented mortality reduction at 48 h with no difference between evaluations at 48 and 72 h, whereas the control treatment with Btk presented the highest mortality at 48 h. Still, regarding the Stubble-Aid®, the control treatment with Btk presented higher percentage of dead larvae at 72 h, unlike 2RC + Btk which the highest percentage was observed at 48 h, while in concentrations ½ RC + Btk and RC + Btk no difference between 48 and 72 h was observed.

Matan Plus at the RC + Btk and 2RC + Btk presented higher mortality rate at 48 h, whereas the control with Btk and the product at ½ RC + Btk showed no significant difference between 48 and 72 h.

Most products affected negatively the CFU/mL, but only Bordeaux mixture inhibited crystals toxicity. Bordeaux mixture (pH $\approx$ 12) was the only product with pH above 7.5, and the only that impaired Btk ability to kill caterpillars, therefore the pH can explain this result. According to Batista Filho et al. (1998) pH values above 7 and near 11 are detrimental to the Bt. The lack of crystals toxicity after incubation with Bordeaux mixture may be related to crystal dissolution in alkaline medium not fully activating or even deactivating proteins.

Table 4. Total percentage means (± SE) of *A.gemmatalis* larvae mortality caused by *B. thuringiensis* subsp. *kurstaki* HD-1 at 12, 24, 48 and 72 hours after incubation (30 ± 2 ºC, 150 rpm, 2 h) with sterile distilled water and alternative products at different concentrations and initial and final pH.

Treatment	Time					pH	
	12h	24h	48h	72h	Total mortality (%)[1]	0h	2h
Agro-Mos®							
Control Water	0.0±0.00Aa	0.8±0.80Aa	0.0±0.00Da	0.8±0.80Ba	1.6±0.98B	6.22	6.37
Control ½RC	0.0±0.00Aa	0.8±0.80Aa	0.0±0.00Da	0.8±0.80Ba	1.6±1.60B	3.63	3.55
Control RC	0.0±0.00Aa	0.8±0.80Aa	2.4±1.60CDa	0.0±0.00Ba	3.2±1.96B	3.50	3.45
Control 2RC	0.0±0.00Aa	0.0±0.00Aa	0.0±0.00Da	0.0±0.00Ba	0.0±0.91B	3.34	3.32
Control Btk	0.0±0.00Ac	0.8±0.80Ac	36.4±5.20Ab	58.8±5.63Aa	96.0±1.79A	5.24	5.38
½RC + Btk	0.8±0.80Ac	0.0±0.00Ac	24.8±4.80ABb	65.6±5.15Aa	91.1±2.94A	3.89	3.88
RC + Btk	1.6±1.60Ac	0.8±0.80Ac	12.3±5.76BCb	56.0±14.03Aa	70.7±10.08A	3.79	3.73
2RC + Btk	3.2±1.96Ac	0.8±0.80Ac	20.8±5.99ABb	66.4±4.83Aa	91.2±4.08A	3.61	3.54
CV 1 (%) =	37.3	CV 2 (%) =	43.5	CV 3 (%) =	16.5		
Biogermex							
Control Water	0.0±0.00Aa	0.0±0.00Aa	0.0±0.00Ca	0.0±0.00Ca	0.0±0.00B	6.22	6.37
Control ½RC	0.0±0.00Aa	0.0±0.00Aa	0.8±0.77Ca	1.5±0.92Ca	2.3±0.92B	3.93	3.99
Control RC	0.0±0.00Aa	0.0±0.00Aa	0.0±0.00Ca	0.0±0.00Ca	0.0±0.00B	3.89	4.01
Control 2RC	0.0±0.00Aa	0.8±0.80Aa	0.0±0.00Ca	4.8±3.88BCa	5.6±3.71B	3.51	3.50
Control Btk	0.7±0.67Ab	4.6±2.04Ab	88.7±4.07Aa	6.0±4.85BCb	100.0±0.00A	5.24	5.38
½RC + Btk	2.3±1.58Abc	5.4±2.74Abc	81.3±3.68Aa	11.0±4.40Bb	100.0±0.00A	4.34	4.47
RC + Btk	2.4±1.00Ac	3.2±1.96Ac	67.9A±3.53Ba	25.7±3.09Ab	99.2±0.76A	4.04	4.09
2RC + Btk	0.8±0.80Ab	3.2±1.55Ab	51.7±6.19Ba	43.3±6.93Aa	99.2±0.76A	3.77	3.81
CV 1 (%) =	26.2	CV 2 (%) =	35.9	CV 3 (%) =	11.8		
Bovemax							
Control Water	0.0±0.00Aa	0.0±0.00Aa	0.0±0.00Ca	0.0±0.00Ba	0.0±0.00B	6.22	6.37
Control ½RC	0.8±0.83Aa	0.0±0.00Aa	0.0±0.00Ca	0.0±0.00Ba	0.8±0.84B	5.55	5.54
Control RC	0.8±0.80Aa	0.0±0.00Aa	0.0±0.00Ca	0.0±0.00Ba	0.8±0.80B	5.65	5.32
Control 2RC	0.0±0.00Aa	1.0±0.95Aa	3.6±2.20Ca	0.0±0.00Ba	4.5±2.93B	5.63	5.73
Control Btk	0.7±0.67Ab	4.6±2.04Ab	88.7±4.07Aa	6.0±4.85Bb	100±0.00A	5.24	5.38
½RC + Btk	3.1±0.78Ab	1.5±1.48Ab	44.1±10.3Ba	43.8±7.20Aa	92.5±4.20A	5.43	5.52
RC + Btk	2.4±1.59Ab	1.6±0.96Ab	43.5±13.68Ba	45.3±11.56Aa	92.8±3.13A	5.80	5.83
2RC + Btk	6.6±2.54Ab	2.5±1.62Ab	46.9±8.34Ba	32.8±6.01Aa	88.8±6.08A	5.39	5.54
CV 1 (%) =	45.4	CV 2 (%) =	44.9	CV 3 (%) =	13.1		
Bordeaux mixture							
Control Water	0.0±0.00Aa	0.0±0.00Aa	0.0±0.00Ba	1.0±1.00BCa	1.0±1.00B	6.22	6.37
Control ½RC	0.0±0.00Aa	0.0±0.00Aa	0.0±0.00Ba	1.0±0.96BCa	1.0±0.96B	12.54	12.13
Control RC	0.0±0.00Aa	0.0±0.00Aa	0.0±0.00Ba	0.0±0.00Ca	0.0±0.00B	12.62	12.25
Control 2RC	0.0±0.00Ab	0.0±0.00Ab	0.0±0.00Bb	2.8±1.17ABa	2.8±2.82B	12.63	12.27
Control Btk	0.0±0.00Ac	0.0±0.00Ac	88.7±6.69Aa	7.6±3.30Ab	96.3±3.70A	5.24	5.38
½RC + Btk	0.0±0.00Aa	0.0±0.00Aa	0.0±0.00Ba	0.0±0.00Ca	0.0±0.00B	12.25	11.78
RC + Btk	0.0±0.00Aa	0.0±0.00Aa	0.0±0.00Ba	0.0±0.00Ca	0.0±0.00B	12.44	12.08
2RC + Btk	0.0±0.00Aa	0.0±0.00Aa	1.0±1.00Ba	0.9±0.91BCa	1.9±1.17B	12.41	12.24
CV 1 (%) =	29.1	CV 2 (%) =	38.4	CV 3 (%) =	33.5		
Ecolife®							
Control Water	0.0±0.00Aa	0.0±0.00Ba	0.0±0.00Ba	0.0±0.00Ba	0.0±0.00B	6.22	6.37
Control ½RC	0.0±0.00Aa	0.0±0.00Ba	0.0±0.00Ba	0.0±0.00Ba	0.0±0.00B	3.55	3.44
Control RC	0.0±0.00Aa	0.0±0.00Ba	0.0±0.00Ba	2.7±2.73Ba	2.7±2.72B	3.54	3.43

continues

continuation

Control 2RC	0.0±0.00Aa	0.9±0.91Ba	0.9±0.91Ba	0.9±0.87Ba	2.7±1.81B	3.30	3.25
Control Btk	3.9±3.10Ac	7.2±1.32Ac	70.6±3.37Aa	17.4±2.19Ab	99.1±0.86A	5.24	5.38
½RC + Btk	1.6±1.60Ac	3.2±1.50ABc	68.4±5.21Aa	26.0±4.18Ab	99.2±0.80A	4.24	4.35
RC + Btk	3.5±1.83Ac	4.12±1.20ABc	67.2±8.81Aa	19.5±5.30Ab	94.3±3.94A	3.96	4.08
2RC + Btk	0.0±0.00Ac	1.6±0.96ABc	71.1±4.42Aa	25.8±4.62Ab	98.5±0.93A	3.66	3.85
CV 1 (%) =	36.5	CV 2 (%) =	30.8	CV 3 (%) =	11.8		
Matan Plus							
Control Water	0.0±0.00Aa	0.0±0.00Aa	0.0±0.00Ba	0.0±0.00Ca	0.0±0.00C	6.22	6.37
Control ½RC	0.0±0.00Aa	0.0±0.00Aa	0.9±0.91Ba	0.0±0.00Ca	0.9±0.90BC	5.64	5.53
Control RC	0.0±0.00Aa	0.0±0.00Aa	0.0±0.00Ba	0.0±0.00Ca	0.0±0.00C	5.54	5.53
Control 2RC	5.6±3.51Aa	0.0±0.00Aa	0.0±0.00Ba	0.0±0.00Ca	5.6±3.50B	5.27	5.31
Control Btk	3.2±1.50Ab	2.4±0.99Ab	48.1±5.11Aa	41.2±4.29Aa	95.0±2.14A	5.24	5.38
½RC + Btk	1.6±1.60Ab	0.0±0.00Ab	56.1±5.80Aa	39.2±5.75Aa	96.9±1.89A	5.28	5.33
RC + Btk	3.1±1.44Ac	6.2±3.11Ac	65.9±8.25Aa	21.3±7.09Bb	96.5±1.61A	5.27	5.26
2RC + Btk	5.0±1.57Ac	6.1±3.79Ac	60.4±8.59Aa	26.0±4.96ABb	97.5±1.72A	5.26	5.29
CV 1 (%) =	27.9	CV 2 (%) =	34.9	CV 3 (%) =	11.1		
Dalneem							
Control Water	0.0±0.00Aa	0.0±0.00Da	0.0±0.00Ba	0.0±0.00Da	0.0±0.00C	6.22	6.37
Control ½RC	0.0±0.00Aa	0.0±0.00Da	0.0±0.00Ba	0.8±0.80Da	0.8±0.80C	5.05	5.02
Control RC	0.9±0.87Aa	1.1±1.11CDa	0.0±0.00Ba	1.9±1.15Da	3.9±1.70C	4.94	4.94
Control 2RC	2.6±2.61Ab	1.7±1.06BCDb	2.5±1.01Bb	11.3±2.98Ca	18.2±5.95B	4.30	4.30
Control Btk	3.9±3.10Ac	7.2±1.32Abc	70.6±3.37Aa	17.4±2.19BCb	99.1±0.86A	5.24	5.38
½RC + Btk	0.8±0.77Ad	6.1±1.86ABCc	59.3±2.51Aa	33.0±2.95ABb	99.2±0.8A	5.05	5.30
RC + Btk	1.7±1.67Ac	11.1±2.30Ab	66.9±7.94Aa	20.3±7.57BCb	100.0±0.00A	5.04	5.54
2RC + Btk	2.4±1.01Ab	6.5±3.11ABCDb	52.9±4.34Aa	38.2±3.43Aa	100.0±0.00A	5.02	5.33
CV 1 (%) =	29.5	CV 2 (%) =	31.0	CV 3 (%) =	14.3		
Pironim							
Control Water	0.0±0.00Aa	0.0±0.00Ba	0.0±0.00Ba	0.0±0.00Ba	0.0±0.00C	6.22	6.37
Control ½RC	2.3±0.93Aa	0.0±0.00Ba	0.8±0.83Ba	0.0±0.00Ba	3.1±0.79C	3.71	3.64
Control RC	1.7±1.02Aa	0.0±0.00Ba	0.8±0.83Ba	0.0±0.00Ba	2.5±1.66C	3.55	3.54
Control 2RC	3.8±1.11Aa	1.7±1.74Aba	2.6±1.73Ba	3.4±1.46Ba	11.5±3.72B	3.43	3.44
Control Btk	3.9±3.10Ab	7.2±1.32Ab	70.6±3.37Aa	17.4±2.19Ab	99.1±0.86A	5.24	5.38
½RC + Btk	1.5±1.54Ac	3.0±1.82Abc	62.6±11.07Aa	31.5±2.00Ab	98.6±0.88A	3.80	3.95
RC + Btk	0.0±0.00Ac	6.1±2.00Abc	64.8±8.71Aa	25.3±7.48Ab	96.2±2.62A	3.73	4.00
2RC + Btk	3.1±1.93Ac	6.7±2.71Abc	58.3±8.25Aa	29.6±6.98Ab	97.7±2.22A	3.51	3.62
CV 1 (%) =	35.6	CV 2 (%) =	40.2	CV 3 (%) =	10.1		
Stubble-Aid®							
Control Water	0.0±0.00Aa	0.0±0.00Aa	0.0±0.00Ca	0.8±0.80Ca	0.8±0.80B	6.22	6.37
Control ½RC	0.0±0.00Aa	0.0±0.00Aa	0.8±0.80Ca	0.0±0.00Ca	0.8±0.80B	3.45	3.48
Control RC	0.0±0.00Aa	0.8±0.80Aa	0.8±0.80Ca	1.6±0.98Ca	3.2±1.96B	3.33	3.29
Control 2RC	0.0±0.00Aa	0.8±0.80Aa	0.0±0.00Ca	0.0±0.00Ca	0.8±0.80B	3.18	3.13
Control Btk	0.0±0.00Ac	0.8±0.80Ac	36.4±5.20Bb	58.8±5.63Aa	96.0±1.79A	5.24	5.38
½RC + Btk	0.0±0.00Ab	0.7±0.71Ab	48.8±12.57ABa	40.9±8.86Aa	90.4±6.01A	3.89	3.70
RC + Btk	0.0±0.00Ab	2.1±2.09Ab	55.2±6.11ABa	39.5±6.95Aa	96.8±3.20A	3.62	3.60
2RC + Btk	1.6±1.60Ac	3.9±1.76Ac	63.6±7.52Aa	20.4±3.10Bb	89.6±6.65A	3.54	3.66
CV 1 (%) =	28.6	CV 2 (%) =	34.3	CV 3 (%) =	13.2		

Means (± SE) followed by the same uppercase on the line and lowercase on the column in the row do not differ significantly by Tukey test ($P < 0.05$) Product concentration: ½ RC = Half the recommended concentration, RC = Recommended concentration; 2RC = Twice the recommended concentration.

[1] Total percentage of larvae mortality over 72 hours.

Treatment 1 = CV, CV = Time 2, CV = 3 Total mortality.

CV 1 = Treatment; CV 2 = Time; CV 3 = Total mortality.

Source: Elaboration of the authors.

According to Lecadet and De Donder (1967), cited by Habib and Andrade (1998), toxins resulted from crystal hydrolysis from semipurified protease were lethal to larvae of *Pieris brassicae* whereas toxins from dissolution in alkaline media did not show the same effect. According to El-Moursy, Sharaby and Awad (1993) copper sulphate, a substance present in Bordeaux mixture, mixed with Btk crystals and added to the artificial diet of rice moth *Corcyra cephalonica* reduced larvae mortality when compared to those fed diets containing only Btk crystals.

The pH did not seem to have influenced any of the negative effects presented by other treatments because the results were lower than the control treatment with Btk and similar to other treatments that did not affect toxicity.

However, this difference in mortality occurred because both products are insecticides and the higher concentrations tested were toxic to the caterpillars. Bovemax is also an insecticide, based on *B. bassiana* conidia, but no significant mortality was observed in any of the concentrations, according to Alves (1998) fungi require long time to act, therefore the time for conidia to act on the larvae must be longer than 72 h, the period of observation of this study. Still, the studied insect may not be susceptible to the fungus from which Bovemax is made.

Biogermex presented mortality reduction at 48 h in RC + Btk and 2RC + Btk probably due to the presence of any compound of this product, which delayed the action of crystals because no significant difference was observed in total mortality between treatments and control treatment with Btk.

The decision to use an entomopathogen in the field must consider not only the ability to reduce the target insect population, but also the persistence in the environment. Entomopathogens multiply within the host, thus they must be kept active until the target insect is reached (BATISTA FILHO et al., 1998). Therefore, among the tested products, Matan Plus, regardless of concentration, is the most suitable to be used in association with Btk without compromising spores and crystals toxicity.

Conclusions

Except Matan Plus, the other studied natural products affected negatively the CFU/ml formation,. Bordeaux mixture, regardless of concentration, was the only product that affected the crystals toxicity on *A. gemmatalis*.

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